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Gene therapy

MARK A. KAY*[†], DEXI LIU[‡], AND PETER M. HOOGERBRUGGE[§]

*Division of Medical Genetics, Department of Medicine, University of Washington, Seattle, WA 98195; [‡]Department of Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, PA 15261; and [§]Department of Pediatrics, University of Leiden, Leiden, The Netherlands

ABSTRACT   In recent years, there have been a number of technological breakthroughs that have allowed for clinical trials in gene therapy to be initiated. In combination with the genome initiative, the potential for new therapeutics is limitless. Although an enormous amount of information has been obtained in a relatively short period of time, gene therapy is not yet ready for wide-scale practice. Some of the successes and obstacles that remain are summarized in this report.

Gene Therapy: The Problem

Gene therapy can be defined as the introduction of nucleic acids into cells for the purpose of altering the course of a medical condition or disease. In general, with some exceptions, the nucleic acids are DNA molecules encoding gene products or proteins. The original ideas were directed toward treating monogenic (single-gene) disorders, but it has become clear that the gene can be considered a new pharmaceutical agent for treating many types of diseases. Over the last 20 years, the initial thoughts of gene therapy have been transformed into reality with more than 175 clinical trials and 2,000 patients already treated (1). Yet with all the trials, there is still no conclusive evidence for efficacy. Although the expectations have exceeded the initial success of this relatively new field, important information has been gained from preclinical and clinical trials. With this in mind, it is imperative to realize that with recent technological advances, gene therapy for treating a wide variety of diseases is likely to become a reality within the early part of the next century.

There are clearly a number of obstacles limiting successful gene therapy, but the most difficult to overcome has been the inability to transfer the appropriate gene into a target, non-germ-cell tissue, such that an appropriate amount of gene product (usually a protein) is produced to correct the disease. The cell/organism has developed powerful mechanisms to avoid the accumulation of extraneous genetic material. The purpose of this session was to illustrate both the highlights as well as hurdles that remain for developing successful clinical applications.

Vehicles for Gene Transfer

There are two major classes of vehicles for gene transfer: viral and nonviral vectors. This report will concentrate on vectors that have been used in clinical trials (see Table 1). Some researchers believe that viruses will be most successful because they have evolved for millions of years to become efficient vesicles for transferring genetic material into cells, whereas others believe that some of the side-effects of viruses and possible previous exposures rendering the host resistant to transduction (gene transfer into the cell) will preclude their long-term use in gene therapy. Once a vector is designed, two general approaches are used for gene transfer: *ex vivo* where cells are removed, genetically modified, and transplanted back into the same recipient, and *in vivo* therapy accomplished by transfer of genetic materials directly into the patient.

The latter is preferable in most situations, because the complexi of the former method makes it less feasible for wide-sca application.

Viral Vectors for Gene Therapy

Three different classes of viral vectors have been used in clinic trials. The first relates to recombinant retroviruses. To dat retroviruses based on the Mouse Moloney Leukemia virus ha been used most frequently in clinical trials. These vectors a packaged into viral particles, have all viral genes removed b contain some of the viral regulatory sequences, and will on transduce dividing cells. Although efficient at transduction in cells in culture, most cells *in vivo* are quiescent at any point in tim making this vector less useful for *in vivo* therapies unless the cel in a target organ are stimulated to cycle. A second disadvantag of retroviruses is the relatively low concentration of virus that ca be easily produced. More recently, a chimeric Moloney–Huma lentiviral (HIV) vector has been constructed that can transduc at least some quiescent cells *in vivo* including neurons in the brai of rodents (2). This promising advance will require further studic to determine the vector's application in the clinic.

In general, human adenoviruses are responsible for mil illnesses such as upper respiratory infections. The more commo serotypes (2 and 5) have been exploited for use in clinical tria for cystic fibrosis and cancer. This 36-kb double-stranded DN virus contains genes that express more than 50 gene product throughout its life cycle. By eliminating the E1 region of th vector, two goals are accomplished: space is made for placin therapeutic expression sequences and, in the absence of th transactivating E1a protein, the virus cannot replicate. Thus, afte gene transfer, no viral spread will occur. These first-generatio adenoviral vectors were demonstrated to be very efficient a transferring genes into most tissues after *in vivo* administratior In contrast to retroviruses, these vectors can be concentrated t high titer (10e12/ml), can transduce nondividing cells, and do nc integrate into host chromosomal DNA. As a result, these vector were used to temporarily cure a number of animal models witl specific genetic diseases like hemophilia (3) and hypercholester olenemia (4, 5). Enthusiasm for these vectors was tempered by th discovery that low-level production of viral antigens from th vector elicited a robust immunologic response that eliminatec transduced cells and transgene product and/or inhibited repea administration. These studies have prompted a plethora of studie related to vector–host interactions with the hope that the immu nologic barriers could be overcome. Several approaches currenth are being pursued. One includes deleting the viral genes that ar the most immunogenic, and, more recently, clever methods hav been devised to remove all or most of the viral genes (6–8)

Table 1. Gene transfer vehicles

Vector	Advantages	Disadvantages
Viral		
Retrovirus	Integration into host DNA	Semi-random integration
	All viral genes removed	Transduction requires cell division
	Relatively safe	Relatively low titer
Adenovirus	Higher titer	Toxicity
	Efficient transduction of	Immunological response
	nondividing cells *in vitro* and *in vivo*	Prior exposure
Adeno-associated virus	All viral genes removed	Small genome limits size of foreign DNA
	Safe	Labor-intensive production
	Transduction of nondividing cells	Status of genome not fully elucidated
	Stable expression	
Nonviral		
Liposomes	Absence of viral components	Inefficient gene transfer into the nucleus
	Lack of previous immune recognition	Lack of persistence of DNA
		Lack of tissue targeting

ecause of the complexity of the viral genome and toxicity sociated with production of some of the viral proteins at fficient levels to make the virus, removal of these genes has en more difficult than for retroviruses. How well these future-neration adenoviral vectors will function *in vivo* is currently ing determined. Even if the genes are removed, there will be me level of immunity related to transgene production and the ctor capsid itself. Thus, newer immunomodulatory agents that e more selective and are only administered for a brief period ay be useful in blocking these immune responses, as demonrated in several animal models (9).

Recombinant adeno-associated virus (AAV) vectors contain nall, single-stranded DNA genomes and have recently been own to transduce brain (10), skeletal muscle (11, 12), and liver 3) by injection into quiescent tissue or vasculature, feeding the sue in animals. In fact, rAAV has been used to achieve erapeutic (14) or, in some cases, curative (13) concentrations of otting factor IX in mice without toxicity for at least 9 months by vivo delivery. Unfortunately, these vectors have a disadvantage that there is a limit in the amount of DNA that can be ackaged. Thus, larger cDNAs, genes, or complex regulatory cis ements cannot be used with this vector.

Regulation of gene expression may be important for treating me diseases, and recently, several different approaches to gulate gene expression have been used in animal models of ne expression. These approaches include the addition or subuction of small molecules that interact with cis DNA elements nd turn genes on or off. Moreover, tissue-specific regulation can e achieved by using cell-type-specific promoters or by designing ctors that specifically target an organ. Altering the tropism of the vector by constructing new ligands for receptor-specific argeting will certainly be important for future gene therapy applications.

There are a number of additional viral vectors based on pstein–Barr virus, herpes, simian virus 40, papilloma, nonuman lentiviruses, and hepatitis viruses that are currently eing derived in the laboratory. Perhaps these chimeric or as et undiscovered viruses will have properties that offer advantages to clinical gene therapy that are not yet realized.

Nonviral Vectors: Liposomes

Compared with viral vectors, cationic lipid-based delivery ystems have several advantages. Unlike viral vectors, DNA/ipid complexes are easy to prepare and there is no limit to the ize of genes that can be delivered. Because carrier systems ack proteins, they may evoke much less immunogenic reponses. More importantly, the cationic lipid systems have much less risk of generating the infectious form or inducing umorigenic mutations because genes delivered have low nfegration frequency and cannot replicate or recombine.

During the last few years, two classes of cationic lipids have been synthesized and show good transfection activity, which is mostly *in vitro* with established cell lines (15, 16). The first class has two alkyl chains in each cationic lipid molecule, and the other type uses cholesterol as the backbone. Both types of lipid contain either mono- or multiple-amino groups as the cationic function group to form complexes with DNA via electrostatic interactions. Each type of cationic lipid appears to have its preferred cell lines for an optimal transfection activity, even though both types of cationic lipids may show similar levels of transfection activity in a given cell type. With a few exceptions, the transfection activity of these cationic lipids is improved when a helper lipid, dioleoylphosphatidlylethanolamine, is included as part of the liposome composition (17–19). In general, the transfection activity of these cationic liposomes *in vitro* is optimal with slight excess of cationic lipid in the DNA/lipid complexes (17–19).

Despite the early success of cationic lipid systems in transfecting cells mostly *in vitro* or *in vivo* by a local regional administration (20), no solid evidence has been presented in support of their gene transfer efficiency *in vivo* through systemic administration. This is largely due to some technical difficulty in preparing DNA/lipid complexes at concentrations where the injection volume into the animals is not too large and large aggregates will not form. However, such technical problems have been solved to a certain degree. A few recent reports have demonstrated that it is now possible to make a homogeneous solution with a higher concentration of DNA/lipid complexes by using either a much higher cationic-to-DNA ratio (21) than that used in *in vitro* transfection or by including into the lipids nonionic surfactants such as Tween 80 (22, 23) or other polyethylene-based amphiphiles (24). Using rodents, it has been shown that nanogram levels of gene product per milligram of proteins from the tissue extract can be achieved by a single tail vein injection of 25 μg of plasmid DNA containing the luciferase gene as a reporter (21, 25). Among the organs including lung, liver, spleen, heart, and kidney, the level of gene expression is the highest in the lung. Confocal microscopic study of the transfected lung using green fluorescence protein gene as the reporter indicates that the lung endothelial cells are the type of cells transfected (21). Time course shows that gene expression in different organs is transient, with the peak level between 8 and 20 hr and drops to less than 1% of the peak level by day 4. Results from Southern analysis suggest that such transient gene expression is likely due to the degradation of the transgene (21). However, the same level of gene expression can be regained by repeated injection after an interval of 2–3 weeks (21).

Although additional experiments are needed to show that gene expression *in vivo* by an intravenous administration will be useful for therapeutic purposes, these *in vivo* results reconfirm the potential of the lipid system as a carrier for gene therapy. As with

viral vectors, the next challenges are to achieve targeted gene delivery, to control the level of transgene expression, and to devise methods for long-term expression when needed.

Gene Therapy for Hematopoietic Derived Diseases

So far, most clinical trials on gene therapy focus on gene transfer into hematopoietic (blood cells) and cancer cells. In this report, we will illustrate the development of studies on gene transfer into hematopoietic stem cells. All hematopoietic cells arise from a single cell type designated as pluripotent hematopoietic stem cells (PHSCs). Therefore, successful stem cell gene therapy can be applied to a large variety of congenital and acquired blood cell diseases. In the last few years, the cell fraction that includes these PHSCs is being identified using molecules that are present on the cell surface; e.g., it has been found that PHSCs carry the CD34 antigen (CD34+; this is present in approximately 1% of the bone marrow cells) but are negative for other markers ("lin^{neg}, which is the case in approximately 1% of the CD34+ cells). By cell separation techniques, the CD34+lin^{neg} cells can be isolated and used for gene transfer studies. In these stem cell gene transfer studies, the cells are harvested from the patient, gene transfer is performed *ex vivo*, and the transduced cells are subsequently reinfused into the patients. The majority of studies use retroviral vectors as vehicles to mediate gene transfer. Initial trials aiming at gene transfer into hematopoietic cells focused on transfer of the gene encoding adenosine deaminase (ADA) (26–29). Deficiency of ADA results in a T cell defect, leading to a disease called severe combined immune deficiency. Patients with this genetic disease generally die in the first years of life due to overwhelming infections. At the moment these initial gene transfer trials started, gene transfer frequency into stem cells of larger animals such as dogs and monkeys was less than 3% (30–31). Despite this low frequency, clinical trials were initiated, because it was known from bone marrow transplantation experiments that "healthy cells" had a growth advantage over "diseased cells." Therefore, it was hoped that infusion of a low number of genetically repaired cells would result in the outgrowth of a functional immune system. So far, however, the frequency of transduced cells in blood is lower than the 3% reported in larger animal studies. A possible explanation may be that the "human hematopoietic stem cell" is more resistant to transduction than the animal's stem cells. It has also been suggested that, because the lack of pretreatment or conditioning used in animals may affect the outcome of the clinical studies, the transduced cells are only 0.001% of the endogenous stem cells of the patient, which is too low to result in detectable, stable engraftment. However, in similar experiments using animal models, the number of endogenous stem cells was reduced to virtually none by myeloablative therapy. Due to this myeloablation, the proportion of transduced cells following infusion was much higher and resulted in the long-lasting presence of transduced cells (32). Higher-efficiency gene transfer into human hematopoietic stem cells may occur during very long *ex vivo* cultivation of the stem cells over a bone marrow stroma cell layer.

Besides clinical studies in ADA-deficient patients, gene transfer into hematopoietic stem cells has been studied in so-called gene-marking studies (33). These studies are performed in patients who are treated for a malignancy with high-dose cytotoxic therapy, followed by reinfusion of their own stem cells that are harvested earlier in the course of the disease. In these patients, the majority of the graft is reinfused unmodified as a stem cell rescue, but a part of the graft is used for gene-transfer studies with marker genes. Also in these patients, the percentage of genetically marked blood cells after gene transfer is <1%. At present, it is generally believed that

our limited knowledge in basic stem cell biology is the major bottleneck of stem cell gene transfer studies. For example, questions to be addressed include whether or not receptors for viral vectors are expressed and whether it is possible to trigg. stem cells into cycle *ex vivo* without loss of pluripotency.

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